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## Review

Challenges measuring cardiomyocyte renewal<sup>☆</sup>Mark H. Soonpaa, Michael Rubart, Loren J. Field<sup>\*</sup>

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## ABSTRACT

Interventions to effect therapeutic cardiomyocyte renewal have received considerable interest of late. Such interventions, if successful, could give rise to myocardial regeneration in diseased hearts. Regenerative interventions fall into two broad categories, namely approaches based on promoting renewal of pre-existing cardiomyocytes and approaches based on cardiomyogenic stem cell activity. The latter category can be further subdivided into approaches promoting differentiation of endogenous cardiomyogenic stem cells, approaches wherein cardiomyogenic stem cells are harvested, amplified or enriched *ex vivo*, and subsequently engrafted into the heart, and approaches wherein an exogenous stem cell is induced to differentiate *in vitro*, and the resulting cardiomyocytes are engrafted into the heart. There is disagreement in the literature regarding the degree to which cardiomyocyte renewal occurs in the normal and injured heart, the mechanism(s) by which this occurs, and the degree to which therapeutic interventions can enhance regenerative growth. This review discusses several caveats which are encountered when attempting to measure cardiomyocyte renewal *in vivo* which likely contribute, at least in part, to the disagreement regarding the levels at which this occurs in normal, injured and treated hearts. This article is part of a Special Issue entitled: Cardiomyocyte biology: Cardiac pathways of differentiation, metabolism and contraction.

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The regenerative potential of the adult myocardium in cold-blooded animals is well established. Indeed, the initial observation suggesting regenerative growth following injury, in frogs, was reported in 1875 [1]. A substantive body of work, largely by the Rumyantsev (summarized in [2]) and Oberpriller [3–7] laboratories in the 1970s–1980s, further characterized this phenomenon, providing evidence that cardiomyocyte proliferation was a major component of the phenomenon. More recent work established a similar regenerative phenotype in zebrafish [8], a particularly important observation given the suitability of that species for mutational screens. Importantly, the magnitude of cardiomyocyte renewal in lower invertebrates and reptiles is sufficiently high so as to be readily detected by simple histologic analysis at various time points post-injury.

More recent studies examining potential therapeutic interventions to promote cardiac regeneration speak of challenging “the old dogma” that the adult mammalian heart lacks regenerative potential. In fact, the notion of ongoing cardiomyocyte renewal in the mammalian heart is not a new idea. Many studies have examined cardiomyocyte cell cycle activity in normal and injured adult hearts, and the presence of ongoing cardiomyocyte renewal has been long accepted. Rather,

the debate in the literature resides in the magnitude at which this occurs. For example, in the uninjured adult rat heart, values for ventricular cardiomyocyte cell cycle activity (which is often used as a surrogate marker for cardiomyocyte renewal) varied from 0 to 3.15% (reviewed in [9]). This variation likely reflects a number of factors, including the duration of the cell cycle marker being scored. For example, the duration of S-phase is much greater than that of M-phase, and as such assays monitoring cardiomyocyte DNA synthesis (i.e., tritiated thymidine or bromodeoxyuridine incorporation) would by default give rise to a proportionally higher renewal rate than assays monitoring the presence of mitotic figures. Other markers (such as Proliferating Cell Nuclear Antigen or Ki67 immune reactivity) which are expressed throughout much of the cell cycle would give rise to even higher renewal rates.

Another factor contributing to the high level of variation reported for baseline cardiomyocyte cell cycle activity is the accuracy with which cardiomyocytes (and in particular cardiomyocyte nuclei) are identified. It has been argued that the use of confocal microscopy, in combination with immune fluorescence visualization of a cytoplasmic marker (as for example, Troponin T) and a cell membrane marker (as for example, wheat germ agglutinin or laminin) can be used to unequivocally identify cardiomyocyte nuclei in tissue sections [10]. In practice, the z-axis resolution of confocal microscopy is insufficient to resolve non-cardiomyocyte nuclei in close (i.e., <0.5 microns) proximity to cardiomyocyte cytoplasm [11,12], thus precluding accurate cardiomyocyte nucleus identification in some instances. This is

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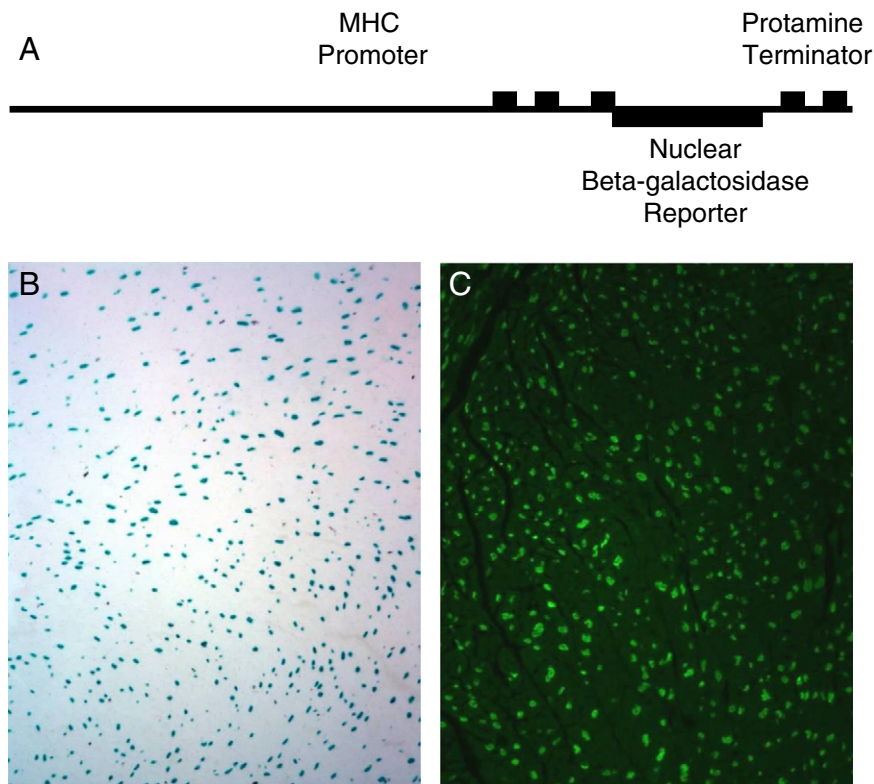
an important consideration, since although cardiomyocytes comprise more than 90% of the mass of the heart, they constitute only 20% of the total cell number.

This caveat was nicely illustrated in experiments using a transgenic mouse wherein the cardiomyocyte-restricted alpha myosin heavy chain promoter [13] targeted expression of a nuclear localized beta-galactosidase reporter (the transgene is depicted in Fig. 1A). These mice (designated MHC-nLAC mice [14]), can be used to identify cardiomyocyte nuclei in tissue sections by simple reaction with a chromogenic beta-galactosidase substrate (i.e., X-GAL; Fig. 1B, blue signal) or by beta-galactosidase immune fluorescence (Fig. 1C, green signal). Sections from these hearts were processed for beta-galactosidase (to identify cardiomyocyte nuclei) and Troponin T (to identify cardiomyocyte cytoplasm) immune reactivity, and stained with wheat germ agglutinin (to identify cell membranes) and Hoechst (to all identify nuclei). The sections were then imaged via confocal microscopy using a high numerical aperture objective, and Z-axis stacks comprising 42 steps at intervals of 0.24  $\mu\text{m}$  for each imaged volume were generated. Observers were supplied with image volumes containing the Troponin T, wheat germ agglutinin and Hoechst signals, and were asked to identify the origin (i.e., cardiomyocyte or non-cardiomyocyte) of each nucleus. These results were then directly compared to those obtained with beta-galactosidase immune fluorescence within the same tissue volume. Significant error rates were observed [15], underscoring the subjectivity of traditional confocal microscopic analyses for cardiomyocyte nuclear identification.

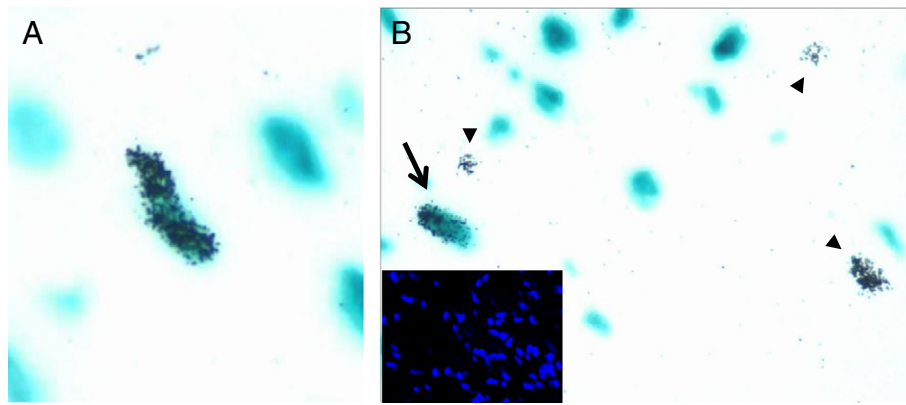
We have used the MHC-nLAC mice to monitor cardiomyocyte cell cycle activity in normal adult hearts. For these experiments, the mice received a single injection of tritiated thymidine, and were sacrificed four hours later. The hearts were harvested, sectioned, and processed

for X-GAL reaction (to identify cardiomyocyte nuclei) and autoradiography (to identify S-phase nuclei). A typical cardiomyocyte in S-phase is shown in Fig. 2A; using this approach only 0.0005% of the cardiomyocyte nuclei in adult hearts were thymidine positive [16]. While this is a relatively low rate of cell cycle activity, it is important to note that it represents only those cardiomyocytes which were synthesizing DNA during the 4 h labeling session. If one assumes a linear relationship, multiplying by 6 would provide a daily cell cycle rate (i.e., 24 h/4 h), and multiplying further by 365 would provide a yearly rate. This would predict a cardiomyocyte renewal rate of 1.09% per year in the uninjured adult mouse heart, assuming that the DNA synthesis events culminated in cytokinesis. This value is remarkably close to the human cardiomyocyte renewal rate calculated by the Frisén laboratory [17]. These later experiments utilized a carbon dating approach that exploited the spike in atmospheric radioactive carbon resulting from numerous above-ground nuclear tests in the 1950s and 1960s, in combination with a cardiomyocyte nuclear-specific marker, and predicted an annual cardiomyocyte renewal rate of 1% per year in young adults.

Monitoring cardiomyocyte DNA synthesis *per se* does not necessarily discriminate between renewal via proliferation of pre-existing cardiomyocytes vs. cardiomyogenic differentiation of stem cells. For example, the human experiment was cumulative in nature, and could detect DNA synthesis events in pre-existing cardiomyocytes as well as in stem cells which subsequently differentiated into cardiomyocytes. In contrast, the labeling period in the mouse experiment was too short to detect *de novo* cardiomyogenic events; it could only detect DNA synthesis events in pre-existing cardiomyocytes. The fact that the normalized mouse data agreed well with the cumulative human data provides circumstantial data suggesting that the primary mechanism



**Fig. 1.** Detection of cardiomyocyte nuclei in MHC-nLAC transgenic mice. A. Schematic depiction of the MHC-nLAC transgene. The MHC promoter consists of 4500 bp of 5' flanking sequence plus Exons 1, 2 and the non-coding region of Exon 3. The protamine terminator was inserted down-stream of the beta-galactosidase sequence to ensure proper processing of transgene-encoded transcripts. B. Low power image of a section from an adult MHC-nLAC transgenic heart following reaction with X-GAL, a chromogenic beta-galactosidase substrate. Cardiomyocyte nuclei appear blue. C. Low power image of a section from an adult MHC-nLAC transgenic heart processed for beta-galactosidase immune reactivity. Cardiomyocyte nuclei are identified by green fluorescence.



**Fig. 2.** Use of tritiated thymidine incorporation in conjunction with the MHC-nLAC reporter transgene to detect cardiomyocyte cell cycle activity in normal and injured hearts. A. Cardiomyocyte DNA synthesis in a normal heart from a mouse receiving a single injection of tritiated thymidine. The image shows a section following X-GAL reaction and autoradiography. An S-phase cardiomyocyte nucleus is identified by the presence of silver grains over blue signal. B. Detection of cardiomyocyte (arrow) and non-cardiomyocyte (arrowheads) DNA synthesis at the peri-infarct zone in a mouse carrying the MHC-nLAC reporter transgene following a single injection of tritiated thymidine. Insert shows the Hoechst epifluorescence signal from the same field.

for cardiomyocyte renewal in the uninjured adult heart is via proliferation of pre-existing cardiomyocytes.

There are also many reports examining cardiomyocyte renewal following myocardial injury in mammalian hearts. Once again, the reported levels of cardiomyocyte cell cycle re-entry, and the degree to which this results in cardiomyocyte renewal, varied greatly between laboratories and appeared to be greatly influenced by the age of the animal at the time of injury, the method used to monitor cell cycle entry, the anatomical position of the cells studied, and the timing of the analyses post-injury (reviewed in [9]). With regards to age at the time of injury, studies in rats from the Gerdes laboratory clearly demonstrated that high levels of cardiomyocyte renewal could be induced during early post-natal life [18], a finding that was recently confirmed by surgical interventions in mice [19]. In mice, this potential for renewal was markedly decreased by post-natal day 7, which coincides with the cessation of developmental cardiomyocyte proliferation in normal mice [20].

With regards to the method used to monitor cardiomyocyte renewal, all of the caveats raised above regarding the nuances of a given cell cycle marker, as well as the identification of cardiomyocyte nuclei, in normal tissue are also applicable when examining injured tissue. Indeed, since injury results in a massive influx of immune cells, as well as a pronounced fibrotic response which is accompanied by the amplification of myofibroblasts, nuclear identification becomes even more problematic. Once again this is nicely illustrated using the MHC-nLAC mice. Fig. 2B shows an image of the peri-infarct zone of an MHC-nLAC mouse which received a single injection of tritiated thymidine 2 weeks following permanent coronary artery ligation. The heart was harvested four hours later and sectioned, and the section was reacted with X-GAL and processed for autoradiography. A single S-phase cardiomyocyte nucleus (arrow) and multiple S-phase non-cardiomyocyte nuclei (arrowheads) are seen. A vast increase in the number of non-myocytes is evident in the Hoechst image from the same field (insert). Retrospective analysis from our laboratory revealed an average border zone (defined arbitrarily as 500 microns from the myocardium/scar interface) cardiomyocyte nuclear labeling index of roughly 0.1% using this approach.

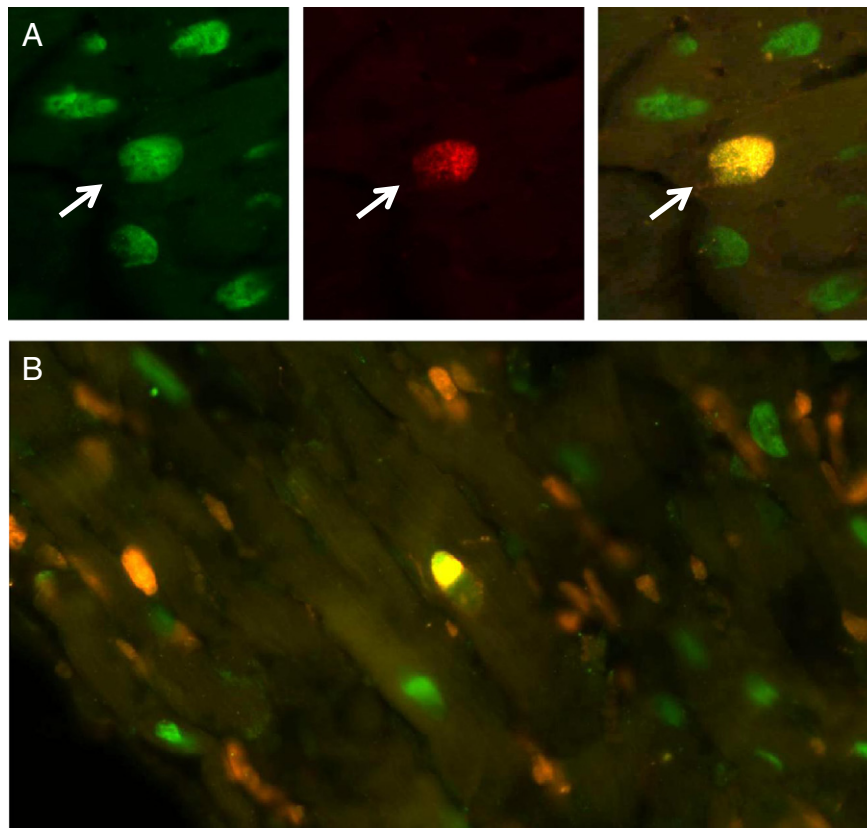
Given the relatively low levels of renewal, analyses based on cumulative label incorporation have the advantage of circumventing errors resulting from sampling size. Bromodeoxyuridine, delivered in drinking water or via implanted osmotic mini-pumps, provides a suitable approach. The MHC-nLAC mice are also useful for the assessment of bromodeoxyuridine incorporation. Fig. 3A shows images from an MHC-nLAC mouse following a single injection of bromodeoxyuridine; the heart was harvested four hours later and sectioned. The sections

were then processed for beta-galactosidase (green signal, left panel) and bromodeoxyuridine (red signal, middle panel) immune reactivity. The same field was also imaged using a dual-channel filter (right panel); the S-phase cardiomyocyte nucleus appears yellow due to the overlay of green beta-galactosidase and red bromodeoxyuridine signals (indicated by the arrow in all three panels). When infarcted MHC-nLAC mice were subjected to cumulative labeling analyses (7 day bromodeoxyuridine infusion using an osmotic mini-pump), a proportionate increase in the cardiomyocyte labeling index at the infarct border zone was observed. However, a dramatic increase in the non-cardiomyocyte labeling index was also observed (Fig. 3B; image was captured with a dual-channel filter, S-phase cardiomyocyte nuclei are yellow, S-phase non-myocyte nuclei are red), further underscoring the need to have a non-subjective assay to identify cardiomyocyte nuclei, particularly when employing cumulative label assays.

Early studies recognized that the elevated level of myocardial renewal observed in injured hearts was largely limited to the region of injury, and that the remote myocardium was not markedly affected (summarized in [2]). Thus, experiments which examine only the infarct and/or a limited proximal region of the border zone will by default report higher renewal rates as compared to experiments which include more distal regions in the analyses. This in turn contributes to variation in the reported renewal rates in injured hearts. The timing of the analyses is also an important consideration, as experiments in rats clearly demonstrated that cell cycle activity reached maximal levels at two weeks following cryoinjury and then rapidly subsided [21]. The degree to which cell cycle activation (as evidenced by S-phase entry) culminates in cytokinesis is also subject to some debate. This is perhaps best illustrated by examples from infarcted human hearts, where markedly different conclusions were drawn based on the inclusion [22] or exclusion [23] of assays to monitor nuclear envelop breakdown. There are two interesting exceptions to the low rates of post-injury cardiomyocyte renewal. The Rumyantsev and Oberpriller laboratories clearly demonstrated atrial cardiomyocyte renewal following injury to the ventricular myocardium in rats [2,24–27]. Furthermore, Rumyantsev clearly documented cell cycle induction in conduction system cells following myocardial injury (summarized in [2]). These findings have not been extensively pursued, presumably because amplification of working ventricular cardiomyocytes is the more relevant clinical target.

As indicated above, short pulse-chase experiments cannot detect stem-cell mediated cardiomyogenic events (unless the resulting cardiomyocytes retain cell cycle activity). In contrast, cumulative label incorporation assays have the potential to detect stem cell mediated events. Indeed, the notion that cardiomyogenic stem cells





**Fig. 3.** Use of bromodeoxyuridine incorporation in conjunction with the MHC-nLAC reporter transgene to detect cardiomyocyte cell cycle activity in normal and injured hearts. A. Cardiomyocyte DNA synthesis in a normal heart from a mouse receiving a single injection of bromodeoxyuridine. The images show the same field of a section following processing for beta-galactosidase (green fluorescence, left panel) and bromodeoxyuridine (red fluorescence, middle panel) immune reactivity. The image in the right panel was captured using a dual-channel filter; the S-phase cardiomyocyte nucleus (indicated by the arrow in all three panels) appears yellow due to the overlay of green and red fluorescence. B. Detection of cardiomyocyte (yellow signal) and non-cardiomyocyte (red signal) DNA synthesis at the peri-infarct zone in a mouse carrying the MHC-nLAC reporter transgene following 7 days of bromodeoxyuridine infusion. Cardiomyocyte nuclei which did not enter S-phase during the labeling period appear green.

persist in the adult heart has received considerable interest over the past ten years. Ironically, the major studies supporting myocardial renewal via extrinsic [28] or cardiac-resident [29] stem cell-mediated cardiomyogenic activity (and which served as a major impetus in promoting the area of research) have proven to be difficult to reproduce, at least by some groups [30–33].

Perhaps the strongest data supporting the presence of cardiomyogenic stem cell activity in the adult heart was generated by the Lee laboratory. These studies used a genetic fate-mapping system comprised of a transgene encoding a conditional, tamoxifen-dependent Cre recombinase under the regulation of the cardiomyocyte-restricted myosin heavy chain promoter in conjunction with a ubiquitously-expressed reporter transgene [34]. Transient exposure to tamoxifen resulted in the generation of adult mice which expressed enhanced green fluorescent protein (EGFP) in terminally differentiated cardiomyocytes, and beta-galactosidase in all other cells. If stem cells undergo a cardiomyogenic event in these animals, it would give rise to beta-galactosidase expressing cardiomyocytes which could be identified in histologic sections. Using this approach, Lee and colleagues were able to demonstrate a progressive increase in beta-galactosidase expressing cardiomyocytes following myocardial infarction. Although these data provide strong evidence for cardiomyogenic stem cell activity, it should be noted that the conversion rate to activate EGFP reporter expression was only ca. 70%, with the remaining adult cardiomyocytes retaining beta-galactosidase activity. Consequently, any differential impact of EGFP vs. beta-galactosidase expression on cardiomyocyte proliferation or survival (i.e., apoptosis sensitivity) would impact interpretation of the data. Unfortunately, this incomplete conversion also renders the

sensitivity of the model too low to detect a 1% annual cardiomyocyte renewal rate, as was reported above for non-injured adult mice.

The utility of genetic tracking systems to identify the origins of “new” cardiomyocytes *in vivo* ultimately is dependent upon the fidelity of the reporter systems. *A priori*, it seems obvious that the more complex the reporter system, the greater the potential for ambiguous results. For example, earlier studies taking advantage of differential folding rates (and consequently differential activation of epifluorescence) of cardiac-restricted EGFP and nuclear red fluorescent protein reporters suggested that zebrafish regeneration resulted via a stem cell-based mechanism [35]. In contrast, data from a simpler reporter system suggested cell cycle-based regeneration [36]; subsequent studies validated this as the main mechanism for myocardial renewal in zebrafish [37]. As indicated above, incomplete Cre-mediated conversion in the bigenic mouse system used by Lee and colleagues complicated data interpretation. Moreover, the suggestion that the MHC promoter may be active in undifferentiated progenitor cells in some studies [38,39] raises further concerns with this approach. Despite these concerns and caveats, genetic tracking systems as described above offer the best hope for establishing the molecular basis for myocardial renewal. The onus is on the investigators to employ the judicious use of controls to establish the limits of their reporter systems, and to restrict data interpretation within these limits.

### Concluding thoughts

The presence of cardiomyocyte renewal in the adult heart has been recognized for over a century. The main disagreements in the

field pertain to the level at which this occurs in the mammalian heart, and the underlying mechanism. It is hoped that this review provides some explanation for the basis of these disagreements. It is encouraging to note that as assays and reagents improve, a consensus with regards to the magnitude of renewal in normal and injured hearts is beginning to emerge. It is also likely that consensus will be reached regarding the underlying mechanism(s) for renewal. In that regard, it is sobering to note that the mechanism of post-injury myocardial renewal in zebrafish (which occurs at a very high level and is easily detected by histology) remained contested for many years.

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